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14. ABSTRACT The purpose of this project is to develop a new process for identifying breast cancer. This process should be at least as convenient as mammography for the patient, more sensitive for detection of the smallest and earliest tumors, and more accurate in distinguishing tumors from normal tissues. First the tumor site is marked by entrapment of a polymer-PNA conjugate due to EPR effect. Based on the longer retention of the polymer conjugate in tumors versus normal tissue, a second polymer-PNA conjugate containing the complementary PNA sequence is administered. This second conjugate will interact with first conjugate due to complementary base pairing to form insoluble and intermolecular polymer networks (microgel). These microgels can then be used as targets to deliver another reagents for imaging. In first two years of grants, we have completed the synthesis of copolymer, polymer conjugates labeled with hilyte or fluorescein dyes and polymer-PNA conjugates. We have also developed a non-invasive method of investigating EPR by skinscan. We have initiated studies with polymer-PNA conjugates.					
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INTRODUCTION

The purpose of this project is to develop a method to detect breast cancer with greatest possible accuracy at smallest possible size. The method should give least stress and inconvenience to the patient. We have proposed to combine different techniques into a three-step process to accomplish this goal.

1. The first goal is to place a “marker” on tumor. For this purpose, we will use the “enhanced permeability and retention (EPR)” effect¹⁻³, which might be universal for all normal tumors. The markers developed herein will be optimized for their ability to accumulate in tumor but not in normal tissues⁴.
2. The second goal is to amplify the signal. For this purpose, multivalent “guest” and a multivalent “host” substance will be used that can form a hydrogel (aggregate/network) of increasing molecular size at the tumor site.
3. The third goal is to detect the signal. For this purpose, we will use the “fluorescence resonance energy transfer” (FRET). FRET approach takes advantage of the “dual probe” feature. There are two fluorescence probes, a donor and an acceptor. The excitation of donor probes results into emission from donor and not acceptor. However, when the probes come close to each other (due to aggregation), there is a transfer of energy from donor to acceptor. The acceptor starts emitting due to resonance energy transfer even though the donor is being excited. FRET phenomenon has been used to investigate aggregation. Working at longest possible wavelengths (near-infrared region) of the spectrum will enhance our detection capabilities.

The original statement of work called for one chemist to initiate studies on the markers during the first year. Use of these markers for biological studies was to be accomplished in the second year. We have moved forward in a balanced manner with both chemistry and biology work. The progress achieved so far is described below.

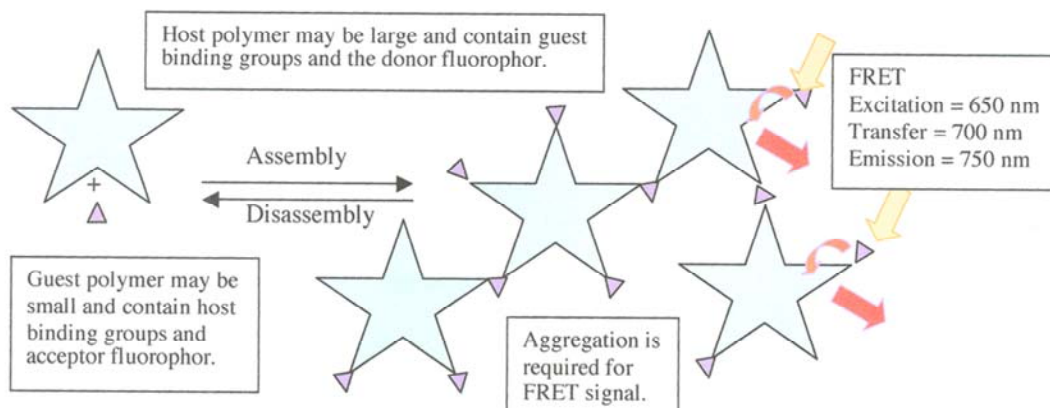


Figure 1. Intermolecular polymer aggregates produced by Schiff base formation between the two polymer strands. The two polymer strands contain donor and acceptor fluorophore that generates FRET signal once the aggregates are formed.

BODY

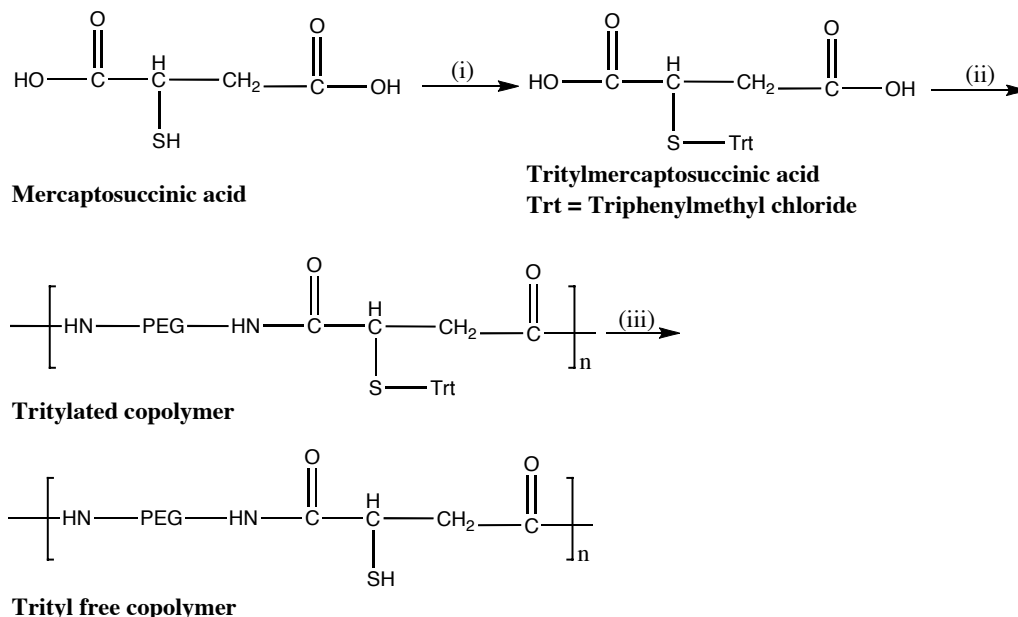
The concept being developed in the present investigation is described above (Figure 1).

Task 1: PREPARATION OF POLYMER

The selection of polymer with optimum molecular size/weight is crucial in accomplishing our aim of forming intermolecular polymer network (microgel) of high molecular weight that stays in tumor. This section describes our work on the synthesis of a copolymer. The commercially available polymers that are being evaluated for this work along with in house prepared copolymer are also described below.

Task 1a. Poly[poly(ethylene glycol)-alt-(polymercaptosuccinic acid)] copolymer

The original idea was to prepare a “host” polymer and a “guest” polymer as shown below. These polymers will accumulate mostly in tumors and not in the normal tissues due to “Enhanced Permeability and Retention (EPR)”. These polymers will recognize each other (by chemoselectivity) and spontaneously form a network (microgel) in the tumors (Figure 1). The host and guest copolymer will each have a donor and an acceptor fluorophore for generating fluorescence resonance energy transfer (FRET) signal.



Reagents and Conditions: Trityl chloride / DMF; (ii) DiaminoPEG (3.4 kDa), 4-dimethylaminopyridine, p-toluenesulfonic acid, diisopropylcarbodiimide / DCM; (iii) Trifluoroacetic acid / anisole / thioanisole / ethanedithiol.

Figure 2. Copolymer Synthesis.

Copolymer poly[(polyethylene glycol)-alt-(polymercaptosuccinic acid)] was prepared by poly-condensation reaction between polyethylene glycol (PEG)-bis-amine (MW = 3.35 kDa) and tritylmercaptosuccinic acid in dichloromethane (Figure 2). Crude copolymer was obtained after precipitation from cold ether. Copolymer was dissolved in water and low molecular weight impurities were removed by dialysis through Spectra/Por membrane (Molecular Weight Cut Off = 50 kDa). The trityl groups were removed by treatment with trifluoroacetic acid to generate multiple free thiol groups. The thiol-containing copolymer was obtained after precipitation from cold ether. The purity and molecular weight analysis of copolymer was carried out on Water Breeze GPC system equipped with UV and refractive index detector. The transition temperature for copolymer melting (T_m) was estimated as 47.3 °C (Figure 3A). The retention time of purified copolymer was found to be 7.1 min (Figure 3B). The molecular weight of copolymer was estimated as 60 kDa with polydispersity of 1.20. The amount of thiol group present in copolymer was estimated by Ellman's assay (16 μ M per 100 μ g of copolymer).

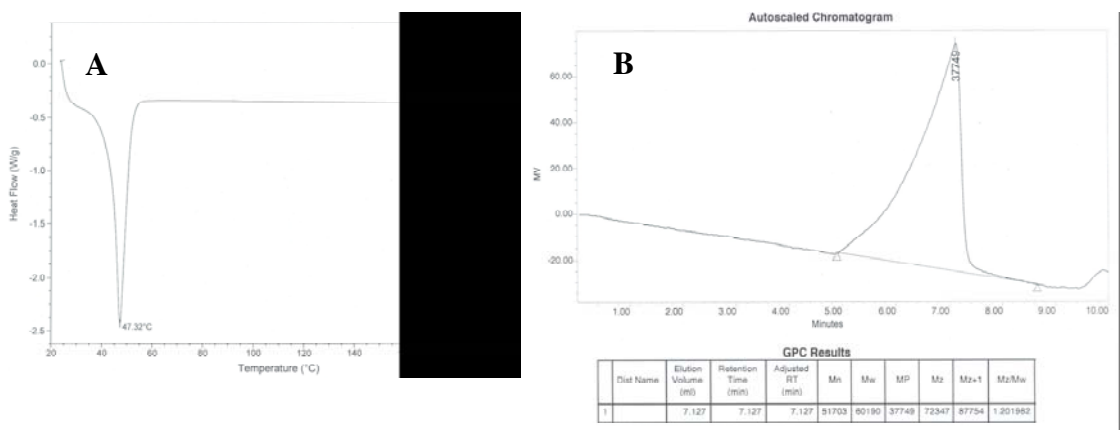


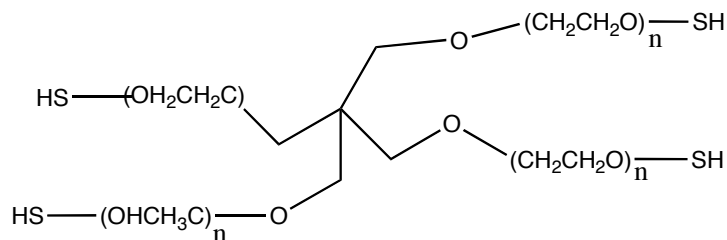
Figure 3. (A) T_m of copolymer; (B) GPC profile and copolymer molecular weight estimation.

The reason for using this particular copolymer is that the free amino terminus is utilized for attachment to donor or acceptor fluorescence moieties and the free thiol groups are used for attachment to complementary functional moieties, amine and aldehyde for the formation of Schiff's base. However, we are using PNA-PNA hybridization to form intermolecular network, further functionalization with amine and/or aminooxy group is not required. The ability of copolymer to form intermolecular networks was evaluated *in vitro* by using commercially available cross-linkers, 1, 6-hexane-bis-vinylsulfone (HBVS) and 1, 11-bis-maleimidotriethyleneglycol (BM[PEO]₃). A 4% solution of copolymer (w/v) formed hydrogel in ~4 min with HBVS and less than ~1 min with BM(PEO)₃ in sodium phosphate buffer at pH 7.4. The stoichiometry, percentage copolymer (w/v) and pH and molality of buffer were varied to investigate optimum conditions required for hydrogel formation.

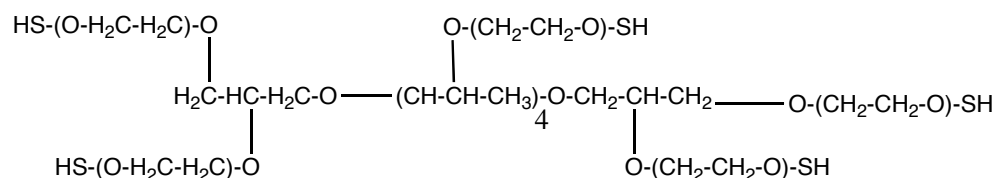
Task 1b. NOF polymers

The copolymer prepared herein has molecular weight of 60 kDa. Polyethylene glycol derivatives behave like molecules of higher molecular sizes as compared to proteins of same molecular weight due to their characteristics hydrodynamics properties. Polymers accumulate in tumor depending on their molecular size. Low molecular weight polymers accumulate in tumor fast but are also cleared quickly. Comparatively high molecular weight polymers (20-30 kDa) accumulate in tumor but are cleared slowly. Polymer with very high molecular weight may therefore be slow to accumulate (See below for additional evidence). The present plan is to work with polymers in the molecular weight range that are quick to accumulate in tumors and can then be aggregated to form a intermolecular network (microgel) of high molecular weight /size so that they stay in tumors. It was therefore decided to evaluate multiple thiol containing polymers available from NOF America Corporation in the molecular weight range of 10-40 kDa along with the copolymer (Figure 4). These polymers contain 1-8 thiol groups that can be used for further modification. Polymers supplied by NOF are analytically pure, have low poly-dispersity and can be analyzed by GPC as well

as MALDI-TOF. Following polymers were obtained and analyzed for this study (Figure 5).



4-Arm Thiol Polymer (10 kDa and 20 kDa)



8-Arm Thiol Polymer (20 kDa)

Figure 4. Poly(ethylene glycol) polymers containing multiple thiol groups obtained from NOF.

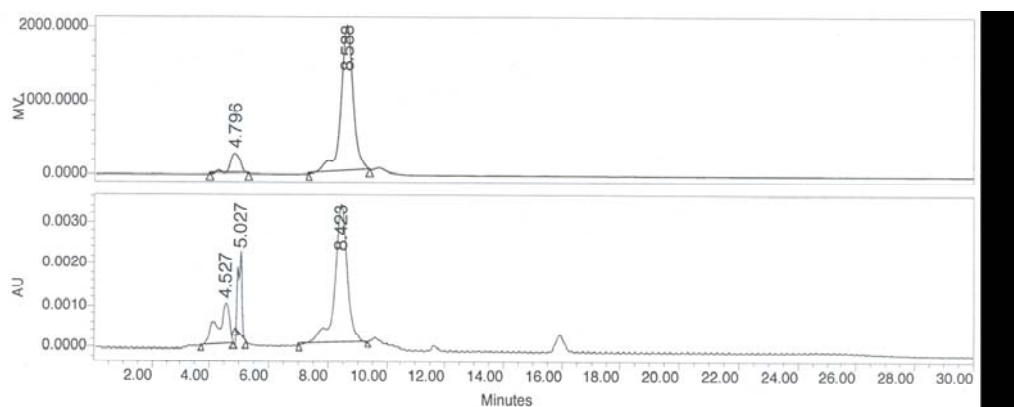


Figure 5. Gel-permeation chromatogram of 4-arm thiol polymer (20 kDa) used in present study.

Conclusion: Polymer of different molecular size (3.4 kDa-40 kDa) has been obtained and high-molecular weight copolymer (60 kDa) has been prepared in the laboratory. The procedure is now standardized and it is reproducible.

2. ENHANCED PERMEABILITY AND RETENTION (EPR)

The vessel characteristics of tumors enhance the permeability of blood vessels in tumor tissues to macromolecular compounds like plasma proteins, macromolecular drug and lipidic microparticles. The impaired clearance of macromolecules from the interstitial space of tumor tissue contributes to retention of these molecules in tumor for long periods. This is called “Enhanced Permeability and Retention (EPR)”¹. Thus, EPR results in accumulation of biocompatible macromolecules in much higher concentration than those in normal tissues and organs, even higher than those in plasma. The EPR effect can be observed for proteins with molecular weight greater than 50 kDa. The

molecular size of proteins cannot however be related to polyethylene glycol polymers because proteins possess a very compact and globular structure whereas polymers display a coiled/extended conformation⁵. Poly(ethylene glycol) polymers has higher molecular volume as compared to proteins with same molecular weight due to random conformation and ability to coordinate water molecules (three per monomeric unit). This water cloud gives high hydrodynamic volume to PEG that makes it difficult to compare sizes among proteins, polymers or polymer conjugates. The selection of polymer molecule with appropriate molecular size is crucial for accomplishment of our goals. The polymer with too large molecular size accumulates in tumors slowly. Polymers with low molecular weights accumulate faster in tumors but are also cleared equally faster. Polymers with molecular size that accumulate quickly in tumors but stays long enough to be able to form intermolecular polymer network of high molecular size should be the most suitable for this study.

Task 2a. Preparation of polymers labeled with fluorophores

Fluorescently labeled polymers of different molecular weights/sizes were prepared to investigate EPR. This work will be completed soon (~2 months). This will lead us to the selection of polymer with optimum molecular size for imaging experiment. Besides, we are employing non-invasive skin scan technique to detect fluorescently labeled polymers and therefore EPR. This will lead us to new method of monitoring and detecting EPR in tumors by non-invasive monitoring of fluorescence.

Hilyte labeled PEG polymers (MW = 3.4 and 20 kDa) and copolymer (MW = 60 kDa) was prepared (Figure 6). This was accomplished by reacting excess of Hilyte succinimidyl ester with polyethylene glycol amine. The excess of Hilyte probe was removed by dialysis. The Hilyte labeled polymers were obtained after freeze-drying. The Hilyte labeled copolymer was prepared by using Hilyte maleimide instead of succinimidyl derivative. Hilyte dye emits in near infra red (NIR) region of spectrum. The excitation wavelength is 754 nm whereas the emission wavelength is 778 nm. The measurement in NIR region of spectrum helps to exclude contributions arising from auto fluorescence of cells/tissues. The detection sensitivity was found to be very poor and fluorescence intensity was relatively low due to self-quenching of Hilyte fluorescence (See EPR studies below).

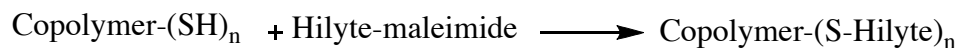
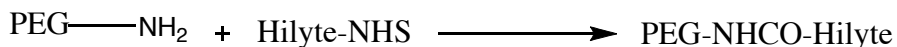


Figure 6. Preparation of PEG-Hilyte (20 kDa) and Copolymer-Hilyte.

It has been reported that for visualization of surface tumors, brightness of fluorophore (high quantum yield and molar extinction coefficient) is more important than long-wavelength excitability⁶. Consequently, more fluorescently labeled polymer conjugates were prepared by using fluorescein instead of Hilyte (Figure 7). Fluorescein is one of the most used fluorophore with excitation and

emission wavelengths of 480 nm and 515 nm respectively. The quantum yield is 0.92 in aqueous sodium hydroxide solution. Polymer conjugates were prepared by reacting PEG dithiol (MW = 3.4 kDa), PEG 4-arm thiol (MW = 10 kDa) and PEG 4-arm thiol (20 kDa) with fluorescein-5-maleimide in buffer or DMF. Centricon membranes or manual size-exclusion chromatography was used to purify these labeled polymer. Products were characterized by MALDI-TOF (Figure 8).

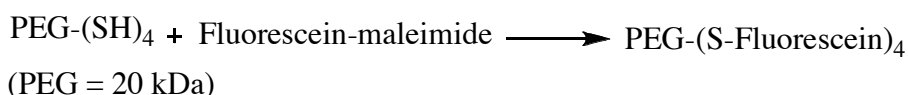
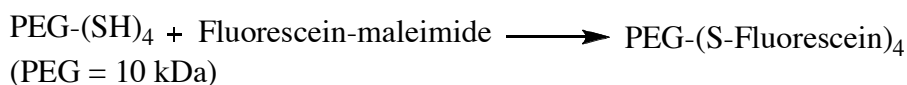
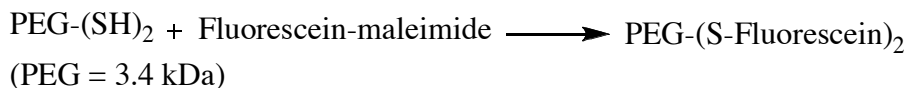


Figure 7. Preparation of polymer-fluorescein conjugates for EPR studies.

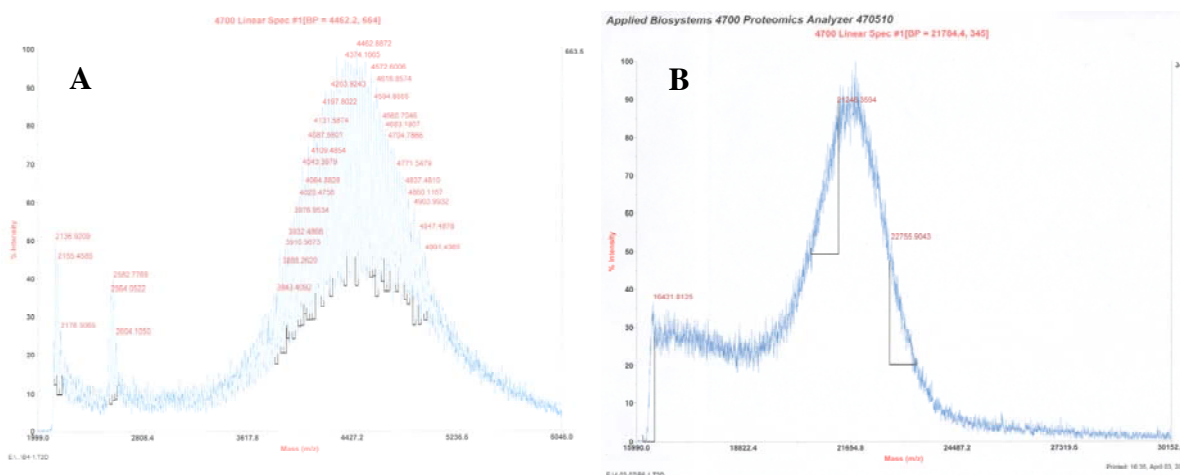


Figure 8. MALDI-TOF analysis of (A) PEG(Fluorescein)₂; (B) PEG(Fluorescein)₄.

Task 2b. EPR studies

Female Balb/c mice of 8 weeks old (Jackson Laboratories) were inoculated with 4T1 cells (10^6 /mouse) via sc into inguinal mammary gland. At 13 days after cell injection, 0.1 ml of polymer-hilyte (3.4 and 30 Kda; 100 mg/ml) and copolymer-hilyte (60 kDa) was injected (i.v.). Skinskan was used to monitor fluorescence of skin or tumor immediately after polymer-hilyte injection (Figure 9). The excitation and emission wavelengths used were 754 nm and 779 nm respectively. EPR effect appeared from 22-28 h after polymer injection for high molecular weight polymers. The fluorescence signal was weak even though the signal/noise ratio was over 2. This was attributed to self-quenching associated with hilyte dye (Figure 10). This was ascertained by examining the standard curve of hilyte dye alone in the concentration range of 0.24-500 mg/ml. When

hilyte concentration exceeded 62.5 mg/ml, fluorescent intensity decreased drastically. However, the fluorescence intensity never went beyond 0.31.

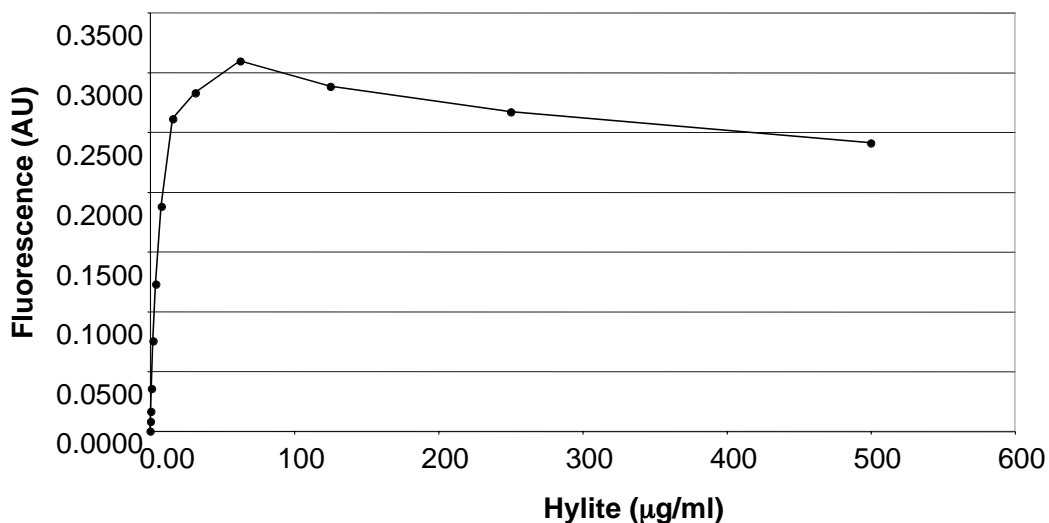


Figure 9. EPR studies with polymer-hilyte conjugate (20 kDa).

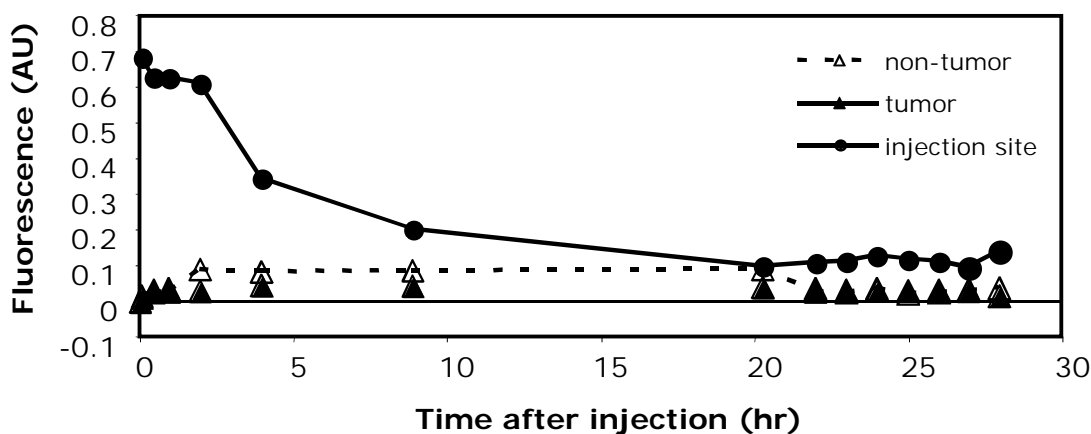


Figure 10. Standard curve for hilyte dye.

EPR studies were therefore carried out with Female Balb/c mice of 8 weeks old (Jackson Laboratories). These were shaved and inoculated with 4T1 cells (10^7 /mouse) via sc into dorsal area. Seven days after cell injection, animals have their hair removed again. One day after hair removal, 0.1 ml polymer-fluorescein (3.4 and 20 Kda; 14 mg/ml) was injected through tail lateral vein. Skinskan (Ex/Em: 480 nm/515 nm) was used to monitor fluorescence of skin or tumor immediately after polymer-fluorescein injection. Fluorescent intensity increased rapidly after polymer-fluorescein injection, exhibiting satisfactory reading compared to hilyte dye, and the high fluorescence value continued till 28 hr without decreasing. The low molecular weight polymer was cleared faster but EPR effect peaked in 1-4 h unlike 22-28 h observed with copolymer-hilyte (60

kDa) after polymer injection (Figure 11). The fluorescence in tumor vs. non-tumor skin was about 2.

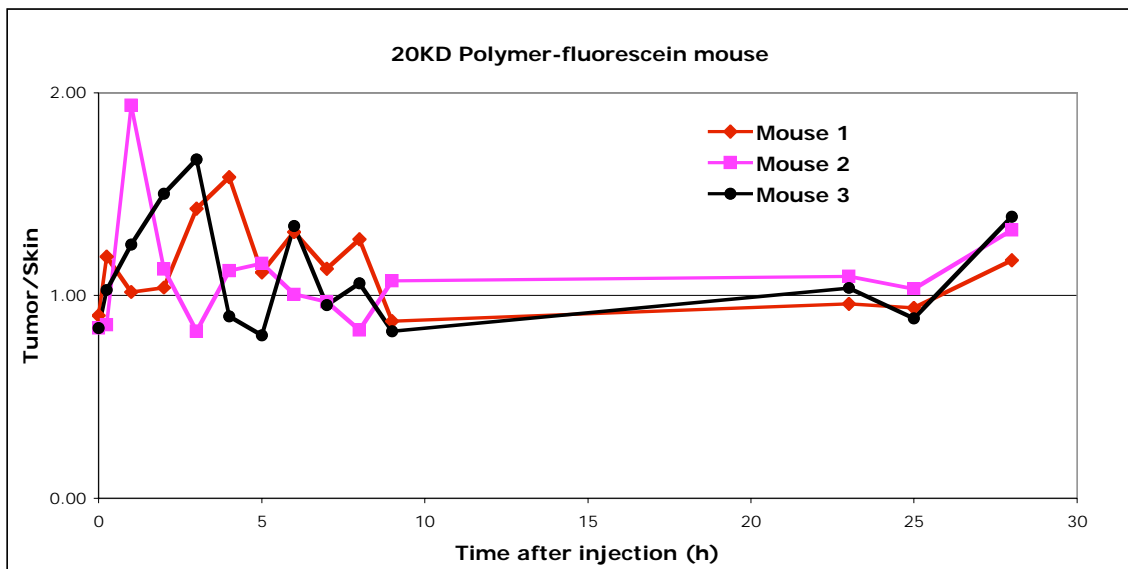


Figure 11. EPR studies using polymer-fluorescein conjugate (20 kDa).

Conclusions: Several labeled polymer conjugates have been prepared. A method based on non-invasive fluorescence detection by skin scan has been developed to investigate enhanced permeability and retention (EPR). This will lead us to optimum molecular weight/size of polymer required for our experiments.

Task 3. POLYMER-PEPTIDE NUCLEIC ACIDS (PNA) CONJUGATES

Original plan was to use chemoselective formation of Schiff's bases to produce intermolecular network in tumor. However, Schiff's bases are reversible in nature and hence must be reduced to stable secondary amines. It was decided to use Peptide Nucleic Acids (PNA) instead to produce intermolecular polymer networks by PNA-PNA hybridization (Figure 12)⁷⁻⁸. PNA's is nucleic acid analog, in which the phosphodiester backbone is replaced with polyamide (peptide) backbone⁹⁻¹¹. PNA's are being increasingly used as genetic tools in place of oligonucleotides because PNA exhibits favorable properties like resistance to nuclease and protease digestion, stability in serum and cell extracts and high affinity for the single or double stranded target sequence. The high affinity for the target sequence is due to the fact that PNA's contain neutral backbone instead of anionic (oligonucleotide) and hence the electrostatic repulsion encountered in DNA-DNA duplex formation is diminished. Consequently, PNA-PNA hybrids are more stable than PNA-DNA hybrids, which in turn are more stable than DNA-DNA hybrids.

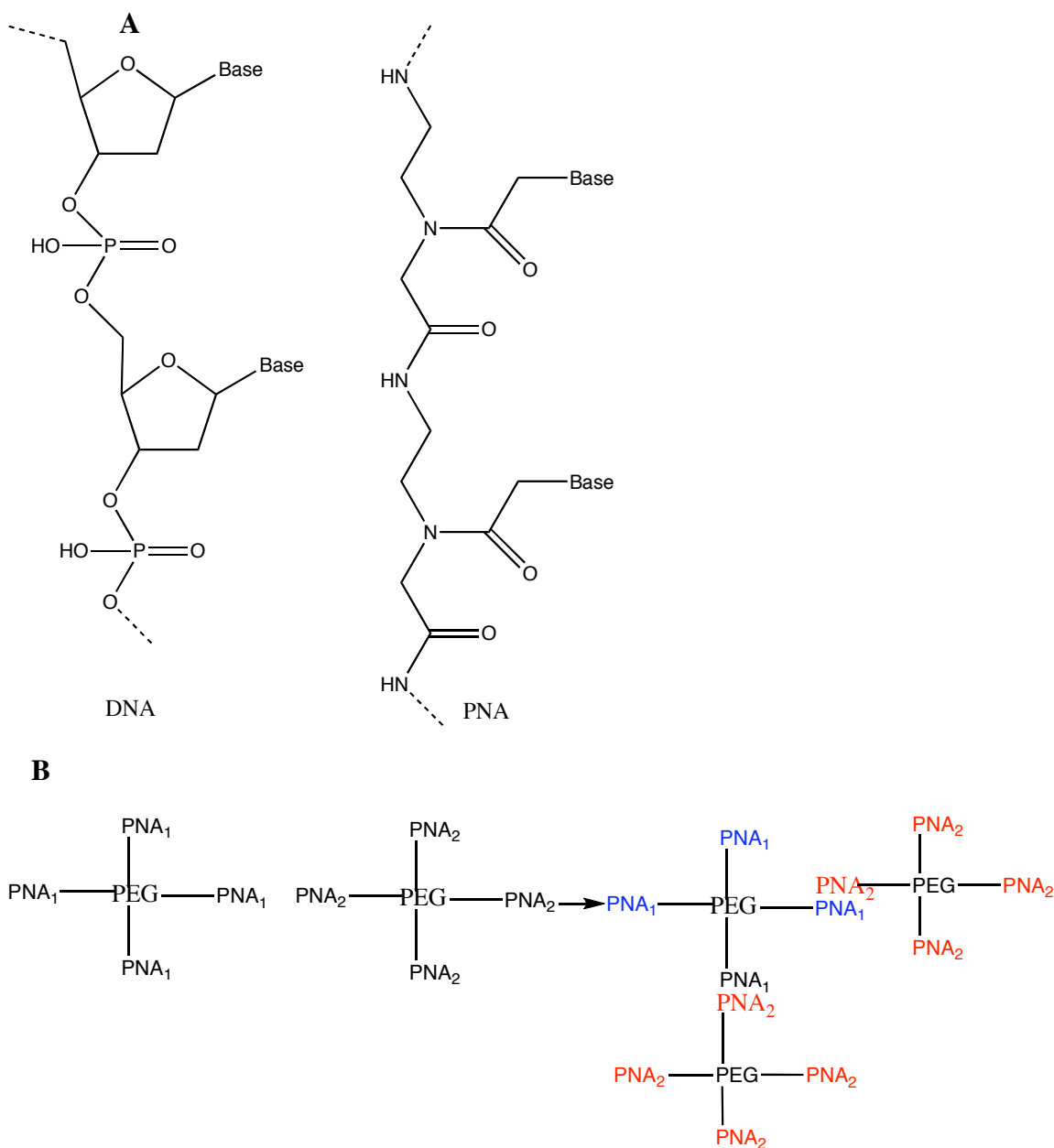


Figure 12. (A) A comparison of DNA and PNA structure; (B) Formation of intermolecular polymer network resulting from PNA-PNA hybridization. The two sequences can be labeled with donor and acceptor fluorophores to generate FRET signals.

Task 3a. Preparation of polymer-PNA conjugates

Following sets of polymer(PNA)₄ conjugates have been prepared. Following three PNA sequences were obtained (Figure 13). Two polymer conjugates were prepared by reacting PNA1 and PNA2 with (polyethylene glycol) 4-arm maleimide polymer (20 kDa) in sodium phosphate buffer (pH = 7.4). The un-reacted PNA sequences were removed by passing through centricon membrane (Figure 14). The reaction was monitored by waters breeze GPC system and Water reverse-phase high performance liquid chromatography.

PNA 1 (SH)-5'-CGT-ATC-ATC-GGA-CTG-3'

PNA 2 Texasred-5'-CAG-TCC-GAT-GAT-ACG-3'-(SH)
(Complementary to PNA1)

PNA 3 5'-CGT-ATC-ATC-GGA-CTG-3'
(un-modified PNA1)

Figure 13. PNA sequences used in present study.

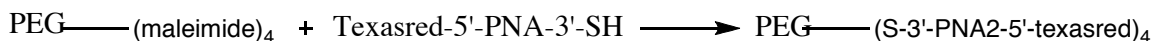
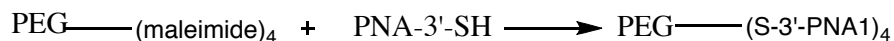
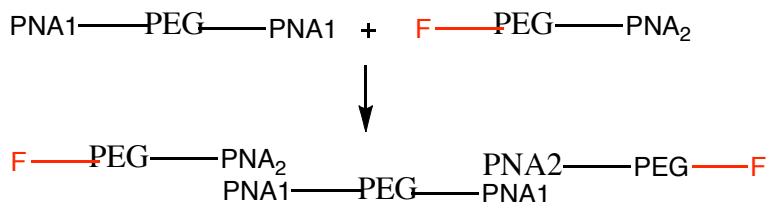


Figure 14. Preparation of polymer(PNA1)₄ and polymer(PNA2)₄ conjugated for present study.

A simplified version is also being investigated. Polymer attached to two similar PNA is prepared. This can be aggregated with fluorescently labeled Polymer-PNA conjugates containing complementary PNA sequence for detection and signal amplification. The concept has been described in figure below (Figure 15).



F = Fluorescein

Figure 15. Preparation of polymer-PNA conjugates and their use in detection and signal amplification.

Task 3b. Injection in mice

Female Balb/c mice of 8 weeks old (Jackson Laboratories) were shaved and inoculated with 4T1 cells (10⁷/mouse) via sc into dorsal area. Seven days after cell injection, animals have their hair removed again. One day after hair removal, 0.1 ml of polymer-PNA-Texas red (20 kDa; 2 mg/ml) was injected through tail lateral vein. Skinskan (Ex/Em: 580 nm/620 nm) was used to monitor fluorescence of skin or tumor immediately after polymer-FITC injection. EPR effect peaked from 1-4 h after polymer injection. The fluorescence in tumor vs non-tumor skin was about 2~3. Fluorescent intensity in both tumor and skin was however very low compared to fluorescein.

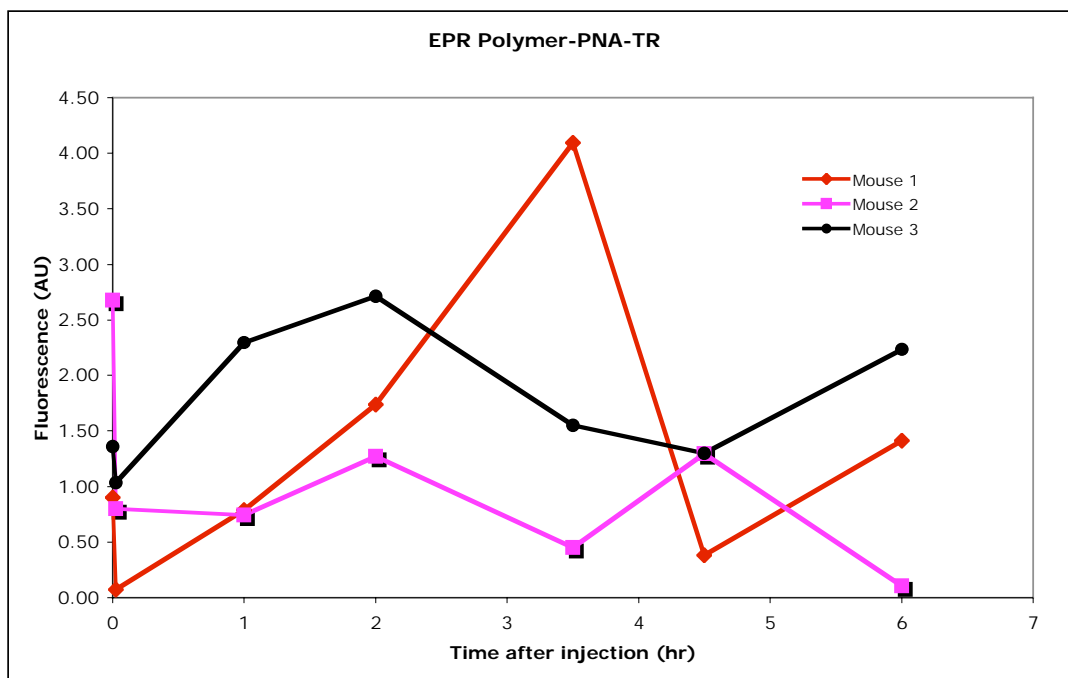


Figure 16. EPR studies using polymer-PNA-texas red conjugate.

Conclusions: Polymer-PNA conjugates have been prepared. The accumulation of conjugates in tumor has been established.

4. SUMMARY

- (a) Copolymer, polymer-hilyte, polymer-fluorescein, polymer-PNA conjugates have been prepared.
- (b) Enhanced permeability and retention studies have been initiated in mice.
- (c) Preliminary EPR studies have been completed with polymer-hilyte and polymer-fluorescein conjugates. More exhaustive studies using larger mice population are ongoing currently.
- (d) A non-invasive method has been developed to investigate EPR by fluorescence detection through skinscan.
- (e) The EPR studies with polymer-PNA conjugates have also been initiated.
- (f) Our efforts will now be directed towards the formation of intermolecular polymer networks in tumors and their detection by dual-probe (FRET) approach.

REPORTABLE OUTCOMES

- (a) The progress so far will lead to thorough investigation of EPR by using fluorescently labeled polymer conjugates.
- (b) This work will lead us to a new non-invasive method of detecting tumors by skin scan.

- (c) Our EPR studies with labeled polymers have been submitted as poster for presentation in 2007 Annual American Association for Pharmaceutical Scientists, November 11-15, San Diego, CA.
- (d) The ongoing exhaustive EPR studies with polymer-fluorescein conjugates (3.4-60 kDa) will be submitted soon for publication (~2 months).

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